Mycol. Res **97** (7): 801–806 (1993) *Printed in Great Britain*

Aggregation of spores and biomass of *Phanerochaete chrysosporium* in liquid culture and the effect of anionic polymers on this process

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The process of pellet formation by *Phanerochaete chrysosporium* in shake-flask culture originated with the aggregation of spores in the early stages of incubation. Spore aggregation was reduced in 0·2% (w/v) Junlon (polyacrylic acid) or 0·2% (w/v) Hostacerin (sodium polyacrylate). The prevention of spore aggregation by polyacrylic acids was a function of polymer concentration, molecular weight and ambient pH. These results indicate that the mechanism of dispersal of fungal biomass in submerged culture by polyacrylic acid is due to a steric stabilization mechanism. However, fermenter cultures of *P. chrysosporium* (and *Aspergillus niger*) in medium containing 0·2% (w/v) Junlon could not sustain the biomass concentrations achieved in equivalent shake-flask cultures. Additionally, fermenter cultures of these two organisms exhibited severe accretion of biomass to the walls of the fermenter and, in the case of *A. niger*, large amounts of foam were produced by cultures in the presence of polymer. This accretion and foaming was not related to inoculum concentration, antifoam type or concentration, or culture volume, but in *A. niger* could be prevented by a fivefold increase in the concentration of mineral salts or trace elements in the medium. Mineral salts supplementation also resulted in attainment of biomass densities similar to those achieved in shake-flask cultures, suggesting that the presence of the polymer in fermenter cultures caused a trace-element limitation not observed in equivalent shake-flask cultures. Junlon and Hostacerin were shown to bind cations present in the growth medium.

The morphology of filamentous fungi in submerged culture ranges from aggregated 'pelleted' growth to dispersed 'filamentous' growth, and in certain species can be altered by variations in nutrient and inoculum concentrations (Byrne & Ward, 1987, 1989). The Basidiomycotina have a strong tendency to aggregate in liquid culture which cannot be controlled by such means, and the resulting growth form can be very heterogeneous, ranging from discrete pellets to single large clumps of biomass in replicate cultures. This obviously makes cultures difficult to monitor and control, and limits the use of such organisms in stirred tank reactors. Growth of pelleted cultures is affected by pellet morphology (Pirt, 1966), there being two extremes. Pellets consisting of densely packed hyphae allow nutrients to enter by diffusion only; they become oxygen-limited at their centre and have only a thin outer growth zone, with the consequence that increase in culture dry weight follows cube-root growth kinetics (Trinci, 1970). On the other hand, if the pellets consist of a loose, open, more filamentous mycelium, agitation of the cultures allows nutrients and oxygen to reach all constituent hyphae and supports exponential growth of the entire biomass. The latter type of growth is also more easily controlled because, in the ideal state, all of the hyphae are growing exponentially and all are in contact with well-stirred medium, so all can respond rapidly to manipulation of the medium. These advantages, though, have to be traded off against increased culture viscosity caused by the filamentous growth. Thus the ability to control the morphology of a fungus in submerged culture is important, since morphology can affect product yield.

Filamentous growth of some moulds in liquid culture has been achieved in aggregating species by the addition of various polymers to the medium. Elmayergi & Scharer (1973), with *Aspergillus niger*, and Byrne & Ward (1987), with *Rhizopus arrhizus*, used the synthetic carboxypolymethylene polymer Carbopol 934. Using the polyacrylic acid Junlon and the sodium polyacrylate Hostacerin, Jones *et al.* (1988*a*) obtained similar results with the basidiomycetes *Phanerochaete chrysosporium* and *Coprinus cinereus*. Many other such compounds have been used successfully to prevent aggregation, but their mode of action is unknown. It has been suggested that some compounds which reduce aggregation may sequester divalent metal ions and so prevent 'salt-bridging' between fungal cells (Byrne & Ward, 1987) or coat fungal cells with an ionized layer which disperses them through electrostatic repulsion (Jones, Moore & Trinci, 1988*b*). These polymers are potentially of great value in that their use promises to open up a wide range of unexploited fungi to commercial use in entirely conventional reactors. Before they can be utilized properly for this purpose their mode of action must be established, but crucial to this is an understanding of the aggregation process itself.

Although the potentially analogous process of yeast

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flocculation has been studied extensively (Beavan *et al.*, 1979; Rahary *et al.*, 1985; Kihn, Masy & Mestdagh, 1988), little work has been done on the mechanism of aggregation in filamentous fungi. Galbraith & Smith (1969) showed that spore aggregation in *A. niger* was an active process which occurred at the time of spore swelling and germ-tube emergence, and that spore clusters subsequently gave rise to pellets.

In the work reported here we have investigated the kinetics of arthrospore aggregation in *Phanerochaete chrysosporium* during the first few hours after inoculation into liquid medium, and assessed the impact of this on subsequent biomass formation in longer-term shake-flask and fermenter cultures.

MATERIALS AND METHODS

Organisms

Phanerochaete chrysosporium Burdsall was kindly supplied by P. M. A. Broda, Department of Biochemistry and Applied Molecular Biology, UMIST. *Aspergillus niger* van Tieghem is maintained in the culture collection of this department. Stock cultures were maintained as slopes at 4 °C using the YPG agar medium described below.

Culture media

The medium used throughout (called YPG) contained (l^{-1}): yeast extract (Oxoid, Basingstoke) 1·5 g; mycological peptone (Oxoid) 5 g; D-glucose (BDH) 10 g. When required, solid medium was prepared by the addition of 1·2% w/v Taiyo powdered agar (Davis Gelatine, Leamington Spa).

The polymers Junlon PW110 and Hostacerin were supplied as anhydrous powders by Honeywell & Stein Ltd, Greenfield House, 69/73 Manor Road, Wallington, Surrey SM6 0BP; unless otherwise stated, they were used at a final concentration in the medium of 0·2% (w/v). Liquid medium containing polymer was prepared by autoclaving nutrient and polymer solutions separately and then combining the two aseptically with thorough mixing. The pH was adjusted by addition of 1 M-NaOH or 1 M-HCl as required. Polyacrylic acids of 5000, 50000 and 500000 M_r, as well as polyacrylamide–acrylic acid copolymer, were obtained from Polysciences Inc., Warrington, PA, U.S.A. They were supplied as aqueous solutions at concentrations from 12·5 to 50% (w/v) and were diluted with distilled water as required.

For shake-flask cultures, 20 ml volumes of medium were dispensed aseptically into 250 ml conical flasks for subsequent inoculations. The technique of Cohen (1973) was used for static cultures: 10 ml portions of medium supplemented with 1 ppm Tween 80 were dispensed into 9 cm diam. plastic Petri dishes for inoculation with arthrospores.

Inoculum

Arthrospores of 2-wk-old cultures of *P. chrysosporium* which had been grown at 25° on YPG agar medium were scraped into sterile distilled water. Suspensions were filtered through four layers of muslin, washed twice with sterile distilled water by centrifugation and their concentrations adjusted to give a final haemocytometer count of *ca* 10^6 arthrospores ml^{-1} in the culture medium.

Monitoring of aggregation

Shake flasks were incubated at 25° on an orbital shaker at 200 rpm. At intervals after inoculation, five samples were removed from the culture to a haemocytometer chamber and counts were made of the total number of fungal particles. Any discrete mass in a culture was considered a 'particle'; particles may consist of a single spore, an aggregation of spores or a fungal 'pellet'. In this context what constituted a pellet depended on the experimental conditions. In the absence of dispersant polymer the biomass was severely aggregated into mycelial clumps, with from one to ten large pellets formed per flask (and accretion of fungal biomass on the flask walls was also common). Successful biomass dispersal by addition of polymeric (or other) medium supplements produced biomass as small pellets of up to 1 mm diam. (and in these cases there was no growth of the organism on the flask walls).

The 'most probable number' method was also used to estimate the total number of colony-forming fungal particles present in shake cultures. One ml samples were removed hourly using wide-bore pipettes, and triplicate serial dilutions to 10^{-7} in YPG medium were performed. The tubes were then incubated at 25° and the pattern of tubes showing growth with dilution was compared to published tables for an estimate of the number of viable particles (Calleja & Johnson, 1977; Theodorou *et al.*, 1990).

In order to determine the increase in size of fungal particles over time, the 'MeasureMouse' graphics system (Analytical Measuring Systems, Pampisford, Cambridge, England) on an Amstrad 1512 PC was used to measure the areas of the profiles of the images of fungal particles as seen in microscope preparations.

Study of aggregate bonding

YPG medium was amended with various concentrations (detailed below) of organic and inorganic compounds and the effect on spore aggregation followed by microscopy and photography of static and shake-flask cultures.

Study of divalent cation binding by Junlon and Hostacerin

A salts solution containing (l^{-1}) $CaCl_2.2H_2O$, 0·1 g; $MgSO_4.7H_2O$, 0·2 g; and $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1·05 mg (the same concentrations as in Vogel's medium) was prepared in double de-ionized water, and 25 ml portions dispensed into glass tubes which had been washed in phosphate-free detergent. Polymer solutions (0·1% (w/v) final concentration) were made up with the salts solution, and 10 ml volumes dispensed into dialysis tubing. The salts solution was used as the hydrating agent because subsequent analysis for metal ions was performed on the bathing medium rather than the

polymer (due to the viscosity of the latter). The pH of the polymer and salts solution were adjusted to 5·8 with 1 M-NaOH or 1 M-HCl. Control treatments had either (polymer-free) double de-ionized water or salts solution in the dialysis tubing. The tubing was then placed in the bathing medium and left overnight at 25°. Five replicates of each treatment were prepared. Subsequent analysis of metal ion concentration in the bathing medium was performed using an atomic absorption spectrophotometer (model 157, Instrumentation Lab Inc., Wilmington, U.S.A.) fitted with a hollow cathode light source. Stock solutions of the three ions were used as internal standards.

Fermenter cultures

All fermentations were carried out in an LH Fermentation 500 series 111 fermenter with a cylindrical, unbaffled, glass vessel of 1 l capacity (LH Fermentation Ltd, Porton House, Vanwall Road, Maidenhead, Berks.). The vessel contents were agitated by a central impeller shaft fitted with two six-bladed paddles mounted at the base of the shaft. The shaft was rotated at 1000 rpm. Aeration was by filtered sterile air (1 ml air ml^{-1} medium min^{-1}) from a remote pump sparged through the base of the impeller shaft; past experience is that this rate and method of air supply avoids oxygen limitation.

The vessel was usually charged with 800 ml of medium; temperature was maintained at 25° by a heating element and chilled cooling finger. Culture pH was monitored by a Russell steam-sterilizable electrode (model ACWL/A200), which controlled the addition of 2 M-NaOH or 1 M-H_2SO_4.

During fermentations, portions of the culture were removed from the vessel through a sampling tube of 4 mm diam., whose open end was approximately 10 mm above the base of the fermenter vessel. The sample (usually 10 ml) was withdrawn using a syringe acting through an in-line air filter.

RESULTS AND DISCUSSION

Aggregation of arthrospores in shake-flask cultures

Results of the assays used to study the aggregation of *P. chrysosporium* arthrospores over the first 10 h of incubation are shown in Fig. 1. The particle number fell by about 85% during this incubation period, while the optical sectional area of particles increased 65-fold. The latter mainly represents the aggregation of spores. Spore swelling and germ-tube emergence only became noticeable from about 7 h onwards, and a plot of haemocytometer particle count against the reciprocal of particle profile area was linear ($r = 0{\cdot}99$). Thus reduction in particle number is initially due to aggregation of spores during the spherical growth stages of germination; it is subsequently exacerbated by entanglement of germ-tubes and accretion to the walls of the culture vessel.

Effects of initial medium pH on the aggregation of P. chrysosporium *spores in shake-flask culture*

Cultures in medium lacking polymer with initial pH values of 5, 6 and 7 all showed a decline in particle number of 91–94% over the 10 h sampling period (Fig. 2), but at initial pH values of 2 and 3 the decline was only *ca* 45% (Fig. 3). Microscopic observation of these latter cultures showed that germ-tube development was poor, indicating that these two pH values were having an inhibitory effect on spore germination and implying that aggregation may depend on changes which occur at the spore surface during germination. After 50 h incubation, little increase in biomass was evident in media of initial pH values 2 and 3; the only visible growth was the formation of a thin rind of mycelium on the wall of the flasks. Within the medium there were a few pellets of less than 1 mm diam. which were composed of clusters of spores with very short germ-tubes. On the other hand, after 50 h incubation, cultures grown in medium with initial pH values of 4 to 7 all had similar culture morphologies, consisting of large clumps of mycelium, a few pellets of up to 6 mm diam., and significant amounts of biomass adhering to the sides of the flasks.

Aggregation of spores of P. chrysosporium *in YPG medium with 0·2% (w/v) polymers*

At initial medium pH values of 5, 6 and 7, media containing Hostacerin showed an approximate linear decline in particle number over the 10 h incubation period immediately following inoculation (Fig. 4), the greatest overall decline in number being in pH 7 medium (77%), but at pH 5 the decline was only 39%. In all these cases the reductions in particle number (i.e. the degree of spore aggregation) were significantly less

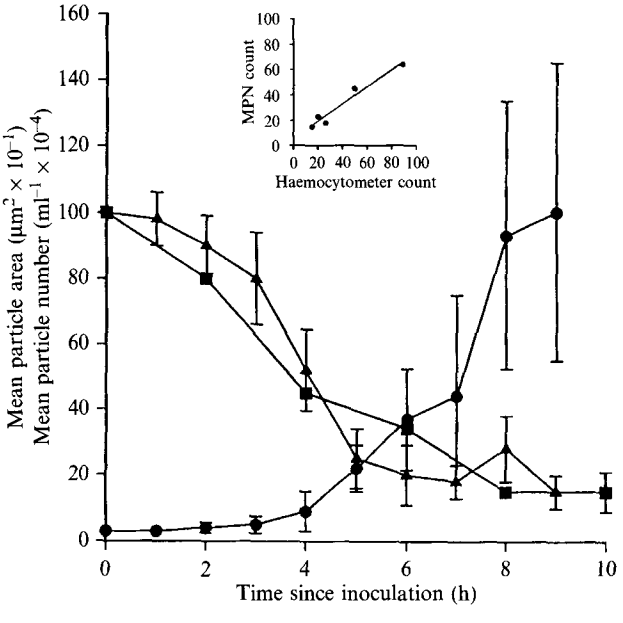

Fig. 1. Change in mean number of fungal particles and mean particle area in shake-flask cultures of *Phanerochaete chrysosporium* grown at 25° in YPG medium during the first 10 h of incubation. Particle number was assessed by haemocytometer (▲) and most probable number (■) counts. Particle areas (●) were measured using an image analysis system. Each datum is the mean of five replicate cultures. Vertical bars represent 95% confidence limits. The inset shows the correlation between haemocytometer counts and counts obtained by the most probable number (MPN) method ($r = 0{\cdot}98$).

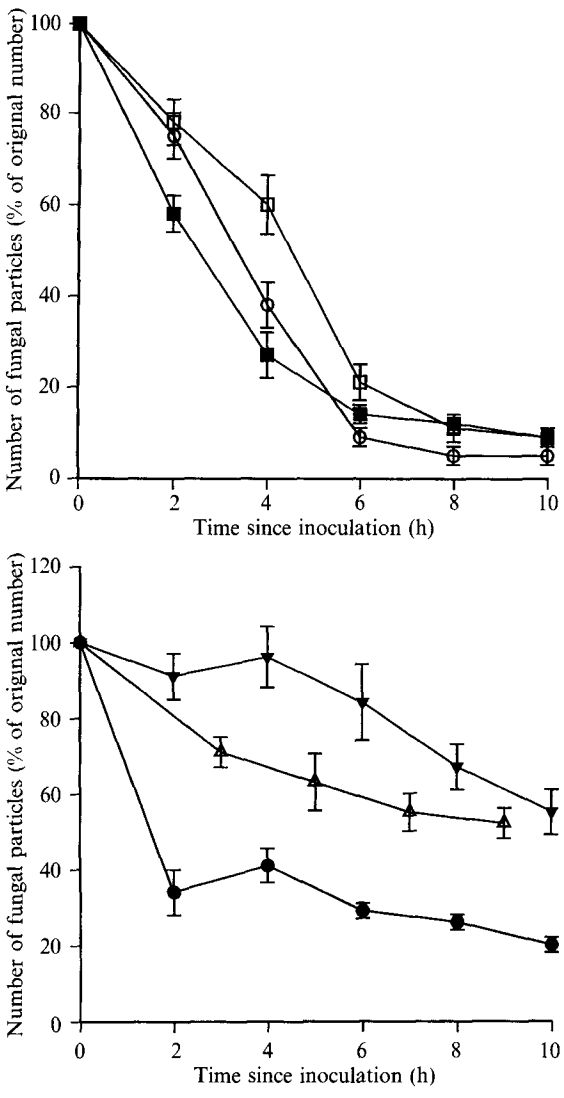

Fig. 2, 3. Effect of pH on the change in mean number of fungal particles of shake-flask cultures of *Phanerochaete chrysosporium* at 25° in YPG medium during the first 10 h of incubation. **Fig. 2.** Initial pH 5 (■), pH 6 (○) and pH 7 (□). **Fig. 3.** Initial pH 2 (▼), pH 3 (△) and pH 4 (●). Vertical bars represent 95% confidence limits.

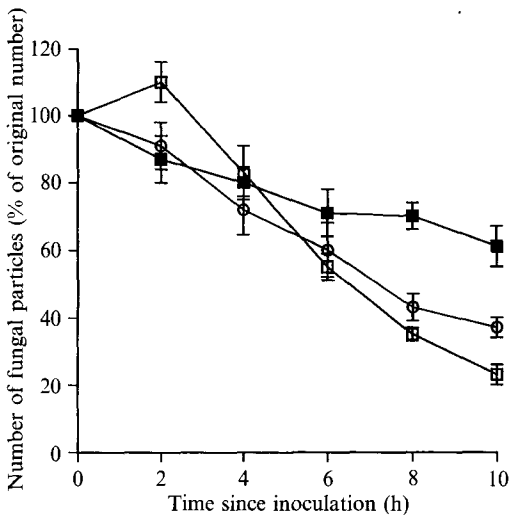

Fig. 4. Change in mean number of fungal particles of shake-flask cultures of *Phanerochaete chrysosporium* at 25° in YPG medium containing 0·2% (w/v) Hostacerin at initial pH 5 (■), pH 6 (○), and pH 7 (□) during the first 10 h of incubation. Vertical bars represent 95% confidence limits.

than occurred in equivalent control treatments. After 50 h incubation, all three media with Hostacerin supported good growth, the cultures having a small pellet morphology (pellets < 1·5 mm diam.) and with little wall growth.

Particle number fluctuated erratically during the initial 10 h of incubation of cultures containing Junlon in medium with initial pH values of 4 and 5 (Fig. 5). At the end of this period, the number of particles in both media had declined by only 25–30%. After 50 h incubation, cultures in these two media showed good growth, with a highly dispersed, filamentous morphology and no wall accretion.

Cultures in medium with initial pH values of 6 and 7 both showed a drop in particle number over the 10 h incubation period of only 15–25% (Fig. 6), but after 50 h incubation very little biomass was obtained, though it had a small pellet morphology.

The observation of Jones *et al.* (1988*b*) that spores become coated with Junlon, and the importance of initial medium pH on the dispersal ability of Junlon described above, together imply that the mechanism by which Junlon prevents biomass aggregation is electrosteric in nature, relying on the polymer coating the biomass and creating repulsive forces between adjacent particles. In medium free of polymer, little aggregation of arthrospores occurred at very acid pH values, suggesting that protonation of some part of the spore outer coat can prevent aggregation. Both polyacrylate polymers were able to mimic this effect at less acid pH values. Junlon was more effective than Hostacerin except that in media adjusted to neutral pH at the outset Junlon greatly reduced growth, though it maintained spore dispersal. One explanation would be that, as well as preventing aggregation, protonation of the outer spore coat somehow inhibits 'germination' (using that term in its widest sense). Thus, in conventional media at pH 2 there was little aggregation and little growth. The polymers are effective because, by coating the spore with a spatially distributed ionic charge, they provide the electrostatic repulsion which limits aggregation without causing charge density to be localized at the spore coat to the extent that germination is adversely affected.

The pH of the medium also affects dispersal by altering the conformation and electrochemical properties of polymers. The poor germination of spores in medium with Junlon at initial pH 6 and 7 might be because the local charge density of polyacrylic acid (Junlon) is so much greater than Na-polyacrylate (Hostacerin) that the presence of Junlon inhibits spore germination in the same way as it is inhibited in medium at pH 2–3 without polymer. On the other hand, this effect could be related to reduced cationic concentrations resulting from chelation by the polymer. The degree of chelation of cations by a polymer is a function of the ambient pH, which affects the degree of expansion of the polymer chain and the viscosity of the polymer solution (Billmeyer, 1971). Overnight dialysis of buffer solutions against both Junlon and Hostacerin (0·1% w/v) did significantly reduce concentrations of Ca^{2+},

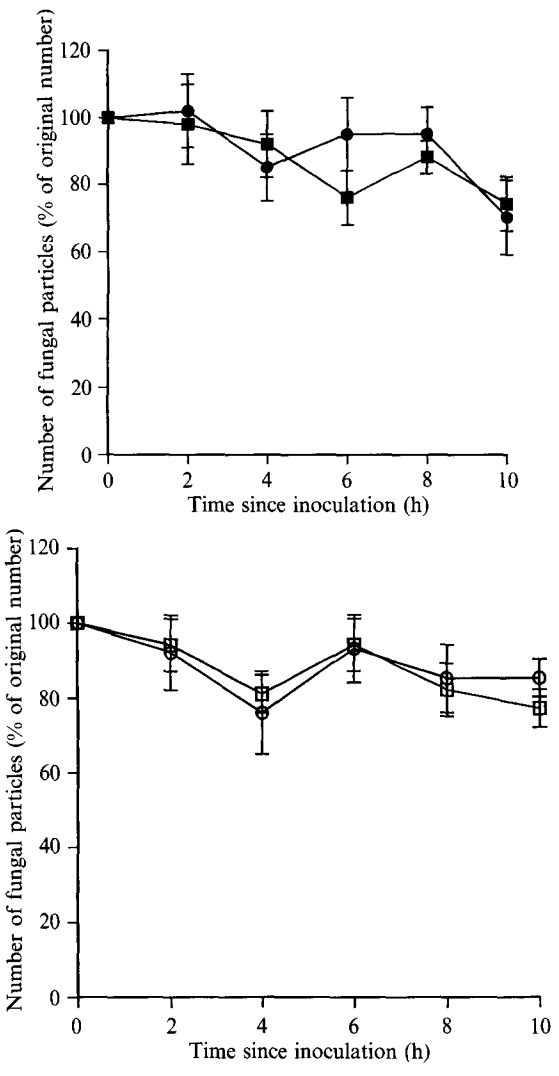

Fig. 5, 6. Effect of pH on the change in mean number of fungal particles of shake-flask cultures of *Phanerochaete chrysosporium* at 25° in YPG containing 0·2% (w/v) Junlon during the first 10 h of incubation. **Fig. 5.** Initial pH 4 (●) and 5 (■). **Fig. 6.** Initial pH 6 (○) and 7 (□). Vertical bars represent 95% confidence limits.

Mg^{2+} and Fe^{2+} in the bathing solution (Table 1). On the other hand, dialysing YPG medium against Junlon through membranes of up to 12000 M_r cut-off had no effect on the morphology of *P. chrysosporium* subsequently grown in the medium in shake-flask culture. This suggests that the dispersal of biomass by Junlon in such cultures is not due to the polymer-sequestering components of the medium, which are necessary for aggregation to occur. It also indicates that oligomers of Junlon of up to 12000 M_r, which were able to migrate out of the dialysis bag, were not the agents responsible for biomass dispersal.

Effect of medium supplementation on the aggregation of P. chrysosporium *arthrospores in stationary liquid culture*

Aggregation of 24-h-old germlings of *P. chrysosporium* in mildly agitated suspensions was prevented by 500 mM and 750 mM (but not 100 mM) concentrations of fructose, galactose, glucose, mannose, methyl-mannoside, sorbose, KCl, $MgSO_4$, NH_4Cl and mannitol. Dispersion was not sustained in longer-term shake-flask cultures; nor in the presence of 5–25 mM dithiothreitol (which interferes with sulphydryl linkages (Rahary *et al.*, 1985)) or 0·5% (w/v) deoxycholic acid (which interferes with hydrogen bonding (Calleja, 1974)). These observations imply that aggregation of *P. chrysosporium* arthrospores is a combination of a number of mechanisms.

The reduction in spore aggregation in the presence of 500–750 mM monosaccharide concentrations could be related to changes in the water activity of the medium, as Humphreys, Matawele & Trinci (1989) found with *Metarhizium anisopliae* and *Paecilomyces farinosus*, which would grow as dispersed filaments rather than aggregates in fermenter culture when the medium contained 440 mM glucose. Humphreys *et al.* (1989) showed that 0·15 M-PEG caused the same reduction in water activity of the medium as 400 mM glucose and had the same effect on morphology of the growing biomass. Unfortunately, *Phanerochaete chrysosporium* did not germinate in the presence of 0·1–0·3 M-PEG, so this proposition could not be tested.

Effects of polyacrylic acids of known molecular weight

Junlon is an industrial-grade product (it is generally used as a thickening agent), which is both chemically impure and of unknown (and probably variable) molecular weight. Tests to investigate the relationship between polymer molecular weight and dispersal of biomass were done using shake-flask cultures and pure polyacrylic acids of 5×10^3, 5×10^4 and 5×10^5 M_r. Both *Aspergillus niger* and *P. chrysosporium* were used to assess organism-specific effects.

P. chrysosporium only grew in media at initial pH 5. Disperse, filamentous growth could not be achieved with this organism in these tests, although a very small pellet morphology was obtained in the presence of 5% (w/v), 10% (w/v) and 15% (w/v) 5×10^4 M_r polyacrylic acid. Dispersal of *A. niger* biomass was achieved using 10% 5×10^4 M_r polyacrylic acid at pH 3 and pH 5, and 2·5%, 5% (w/v), and to a lesser extent 1%, 5×10^5 polyacrylic acid at pH 5. No dispersal of biomass was achieved with 5×10^3 M_r polyacrylic acid at any of the concentrations or pH values tested.

In view of the generally poor results with these oligomers, it is evident that the very highly polymerized polyacrylates are required for effective dispersal at low concentrations of polymer.

Growth in fermenter culture

In the presence of 0·2% (w/v) Junlon, fermenter cultures of *A. niger* and *P. chrysosporium* exhibited severe accretion of biomass to vessel walls and could not attain the same biomass densities as were achieved in equivalent shake-flask cultures. Additionally, fermenter cultures of *A. niger* exhibited severe foaming from the end of the exponential phase in the presence of 0·2% (w/v) Junlon. Biomass accretion was independent of inoculum concentration for both organisms (up to a concentration of 10^6 spores ml^{-1}) and the type or concentration of antifoam used (up to 1% (v/v) polypropyleneglycol with *A. niger*; up to 0·33% (v/v) Foamaster

Table 1. Dialysis of Junlon and Hostacerin against solutions containing known quantities of calcium, iron and magnesium ions

Solutions within dialysis bag	Concentration of cations (μg ml^{-1}) in the solution bathing the dialysis bag		
	Ca^{2+}	Fe^{2+}	Mg^{2+}
Ionic solution (equilibration control)	30·9 ± 0·4	0·1 ± 0·0	17·4 ± 0·7
Distilled water (diffusion control)	22·7 ± 0·6	0·1 ± 0·0	18·0 ± 0·2
0·1% (w/v) Junlon	4·1 ± 0·8	0·1 ± 0·0	6·0 ± 1·0
0·1% (w/v) Hostacerin	14 5 ± 0·6	0·1 ± 0·0	6·0 ± 1·0

Entries show the mean ± S E M. (n = 5) concentrations of calcium, iron and magnesium ions in the bathing solution after overnight dialysis (12000 M_r cut-off) at 25°.

with *P. chrysosporium*). Furthermore, the accretion of *P. chrysosporium* biomass was independent of medium volume and could not be prevented by the addition of additional polymer, nor by treatment of the vessel walls with water-repellent coatings.

Prevention of biomass accretion on the vessel walls, and attainment of biomass densities in the medium comparable to those obtained in shake-flask cultures, was achieved with *A. niger* by increasing the mineral salts concentration of the medium 5-fold.

The fact that fermenter cultures of both organisms in the presence of 0·2% (w/v) Junlon behaved in a totally different manner to equivalent shake-flask cultures implies that the fermenter environment reduces the dispersal efficiency of the polymer. A possible explanation is that the high shear forces within the fermenter broth may detach the polymer from the biomass surface so that a greater proportion of 'free' polymer existed within the medium, causing nutrient limitation by chelation of cations.

Whatever the reason for the difference in behaviour, this is a disappointing outcome. Our experiments indicate that arthrospore aggregation normally occurs rapidly at the onset of the germination process. The indications are that aggregation results from a variety of electrosteric interactions between spore coats, and that polyacrylate polymers can control aggregation by themselves covering the spores with a layer having the same (therefore repulsive) net charge. However, the potential value of this phenomenon will not be realized unless it can be successfully translated to fermenter cultures.

(Accepted 11 November 1992)

We thank SERC for the award of a Research Studentship to M.P.W., and BP International plc for their support.

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